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Rapid Genetic Characterization of Poly(hydroxyalkanoate) Synthase and Its Applications†

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Microorganisms containing short-chain-length (scl-) or medium-chain-length (mcl-) poly(hydroxyalkanoates) (PHAs) are commonly screened by applying rapid staining methods using lipophilic reagents. These methods provide powerful means for general screening of organisms actively producing and accumulating PHAs. The Southern blot hybridization method additionally allows the identification of potential PHA-producing microorganisms. Polymerase chain reaction (PCR)-based detection methods further afford rapid and sensitive means to screen for PHA biosynthesis genes. Specific PCR assays had been developed for the simultaneous or individual detection of the class II mcl-PHA synthase genes of *Pseudomonas*. The amplicons (∼0.54 kb) can be directly sequenced or used as probes for hybridization studies. The sequence information can further be used to initiate chromosome walking for an eventual cloning of the complete PHA biosynthesis operon. In addition, the amplification pattern and sequence data can be used to differentiate subgroups of organisms, as demonstrated for *P. corrugata* and *P. mediterranea*. Other researchers reported PCR methods for the detection of scl-PHA synthase genes and those of *Bacillus* spp., thus greatly expanding the types of PHA synthase gene and the organisms that can be characterized by this approach. The vast sequence information obtainable through PCR-based studies of various PHA synthase operons should facilitate the identification or construction of new PHA synthases capable of synthesizing novel PHAs.

Introduction

Poly(hydroxyalkanoates) (PHAs) are microbial polyoxoesters that have wide-ranging potential applications in the medical, agricultural, and chemical industries. Many bacteria accumulate PHA as carbon and energy reserves in intracellular granular form. As such, most microorganisms synthesize the materials under growth conditions where the carbon source is in excess but one of the nutrients (e.g., nitrogen or phosphate) is limiting. Some bacteria, however, synthesize PHA under nutrient-sufficient conditions.[1−8] On the basis of their monomer composition, the biopolyesters are classified into short chain length (scl-; chain length of the monomers ranging from C3 to C5), medium chain length (mcl-; C6 and longer), and scl-*co*-mcl PHA. The class of PHA synthesized by a bacterium is dictated by the organism's metabolic background and its PHA-biosynthesis genetic composition and function. An appropriate metabolic background ensures the availability of the precursor, and the type of PHA-biosynthesis genes, when expressed, governs the incorporation of monomer of specific chain length. PHA biosynthesis genetic system (*pha* gene locus) is divided into four classes on the basis of the organization of the gene locus and the structure−function properties of the PHA synthase (polymerase) enzymes. The class I *pha* gene locus, as exemplified by that of *Wautersia eutropha* (formerly *Ralstonia eutropha*), consists of *phaC* (coding for PHA synthase), *phaA* (β-ketothiolase), and *phaB* (acetoacetyl-CoA reductase) genes (Figure 1). The class II *pha* operon, found in pseudomonads, comprises two PHA synthase genes (*phaC1* and *phaC2*) flanking a PHA depolymerase gene (*phaZ*). The class III *pha* operon is distinguished by the presence of *phaC* and *phaE* genes that code for the two hetero-subunits of its PHA synthase. Adjacent to the two synthase genes are the *phaA* and *phaB* genes that are transcribed in the opposite direction. The class IV *pha* loci are found in species of *Bacillus* and are constituted of *phaR* and *phaC* coding for the two hetero-subunits of the active PHA synthase and a *phaB* between the two genes.[9−11]

Identification of a PHA-Producing Organism

Lipophilic dyes such as Sudan black B, Nile blue A, and Nile red have been traditionally used for the first-line screening of PHA-containing bacteria.[12−15] Typically, colonies of bacterial cells grown on an agar plate are stained with the lipophilic dye dissolved in an organic solvent. Following a destaining process, cells containing lipid inclusion bodies such as PHA are identified by the retention of the dye. Variations of these techniques that allow for the

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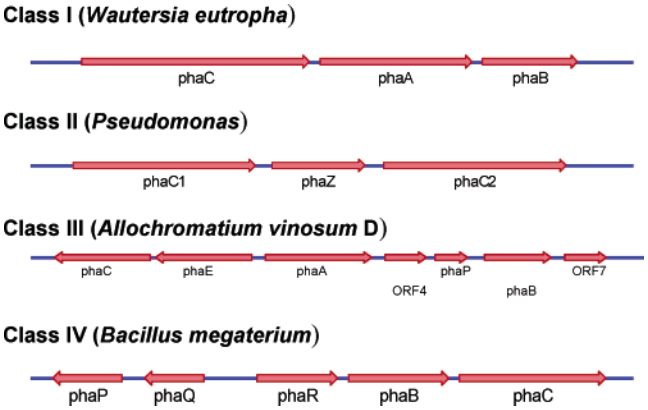

Figure 1. Genetic organizations of the four classes of *pha* loci: *phaA*, β-ketothiolase; *phaB*, acetoacetyl reductase; *phaC* (including *phaC1* and *phaC2)*, PHA synthase; *phaE* and *phaR*, subunit of class III and class IV PHA synthase, respectively; *phaP,* phasin; *phaQ*, homologous to helix-turn-helix multiple antibiotic resistance protein; *phaZ*, PHA depolymerase. ORF, putative open reading frame with an unknown function.

isolation of a viable cell were later developed.[16−17] Because of their nonspecific lipid-binding property, these dye-based methods have also been used to identify cells containing other lipid materials.[16,18−19]

Staining of cells with lipophilic dyes had also been used to facilitate the characterization of PHA-producing cells by instrumentation. Degelau et al.[20] developed fluorescence-based methods to measure PHA levels in cells. In these methods, cells were first stained with Nile red, followed by analysis on either a spectrofluorometer or a laser flow cytometer. The authors reported good correlation between the values obtained with their methods and those yielded by the standard gas chromatography (GC)-based method of Braunegg et al.[21] Similarly, Gorenflo et al.[22] developed a two-dimensional spectrofluorometric method for the quantification of PHA in cells stained with Nile red. Wu et al.[23] took advantage of the shift of the fluorescence maximum between the Nile red stained scl-PHA and the Nile red stained mcl-PHA to differentiate the two types of polymers. The study, however, did not address the occurrence of scl- and mcl-PHA blends or of the scl-*co*-mcl-PHA copolymer. Srienc et al.[24] applied a flow cytometric technique to differentiate microorganisms producing scl- and mcl-PHA copolymers following Nile red staining of the cells. The combination of staining and instrumentation, thus, provide a useful means to identify and even quantify PHA in cells. Certain sample preparations and the specialized instrument, however, are needed in these methods.

Fourier transform infrared (FT-IR) spectroscopy was also investigated as a means to detect PHA in cells. Hong et al.[25] communicated a rapid method to identify and differentiate cells producing scl- or mcl-PHA using FT-IR. A test-screening of several natural isolates using this method resulted in a positive identification of PHA-producing bacteria as confirmed by the standard GC-based method. A method combining FT-IR and multivariative statistical analysis was developed by Kansiz et al.[26] to quantify poly(hydroxybutyrate) (PHB) contents in recombinant *Escherichia coli* cells. Although the method is rapid and sample preparation is minimal, it nevertheless requires the collection of a dedicated data set of FT-IR spectra from the specific PHB-containing organism of interest as a reference. Universal application of the method to other organisms containing different types of PHA had not been demonstrated.

Nucleic Acid Based Methods for Screening of PHA-Producing Microorganisms

A commonly used nucleic acid based approach to screen organisms for a particular gene is the Southern blot hybridization technique. Timm et al.[27] found that the class I *phaC* gene of *W. eutropha* was not useful as a general hybridization probe to identify PHA synthase genes in other bacteria and proceeded to design a 30-mer oligonucleotide for use as a general probe for *pha* genes based on the conserved regions of the PHA synthases of *W. eutropha* and *Pseudomonas oleovorans*. Armed with this probe, these researchers detected and subsequently cloned the class II *pha* gene loci of *Pseudomonas citronellolis*, *Pseudomonas* sp. DSM 1650, and *Pseudomonas mendocina*, and both the class I and class II *pha* loci of *Pseudomonas* sp. GP4BH1. The universal probe was not tested on classes III and IV *pha* genes and likely would not detect these genes anyway by virtue of the basis of their design.

The polymerase chain reaction (PCR) provides a rapid detection of specific genes in organisms. Lopez et al.[28] first reported the use of PCR to identify *phaC*-containing organisms. Because nondegenerate primers derived solely from the *W. eutropha pha* gene sequence were used in their study to identify PHA synthase gene(s) in river-water environmental samples, they obtained a mostly nonspecific PCR product mixture which necessitated a subsequent confirmation of the *phaC* gene by the time-consuming Southern blot hybridization. Solaiman et al.[29] subsequently developed the first PCR procedure that specifically detects class II *pha* genes. The primers were designed on the basis of the highly conserved nucleic acid sequences in regions of the class II *pha* loci of various *Pseudomonas*. Because of the unusually high consensus in these regions, the design of nondegenerate PCR primers (I-179L and I-179R) was possible (Table 1). As can be seen in Figure 2A, the alignment of 32 class II *phaC1* and *phaC2* sequences clearly shows that the binding sites of these primers are located in the exceptionally highly conserved nucleic acid sequence regions of these genes. Screening of various *Pseudomonas* using this method not only verified its general applicability by correctly identifying known mcl-PHA-producing strains but also led to the confirmation of species previously not characterized as accumulating mcl-PHA.[29] A semi-nested PCR protocol was subsequently devised for the selective amplification of a segment of the *phaC1* and *phaC2* isogenes of the class II *pha* locus.[30] In this protocol, one of the primer pairs used in the first-round PCRs (DEV15R for *phaC1* and DEV15L for *phaC2*; Table 1) was designed on the basis of the consensus nucleic acid sequences of *phaZ* genes of various *Pseudomonas*. Figure 2B shows that the binding sites of these primers (DEV15R and DEV15L) are located in the highly conserved regions of the nucleic acid sequences of class II *phaZ* genes. In this protocol, the use of I-179L/DEV15R and DEV15L/I-179R in the first-round PCR would lead to the amplification

Table 1. Primers for PCR-Based *pha* Detection

primer	sequence	ref
phaCF1	ATCAACAA(GGG/A)T(TT/A)CTAC(AA/G) TC(CC/T)T(CC/G)GACCT	33
phaCF2	GT(CCC/GG)TTC(GGG/AA)T(GGG/CC) (AAA/GG)T(CC/G)(TT/A)(CCC/GG) CTGGCGCAACCC	
phaCR4	AGGTAGTTGT(TT/C)GAC(CCC/GG) (AAA/CC)(AAA/CC)(GGG/A) TAG(TTT/G)TCCA	
I-179L(a)	ACAGATCAACAAGTTCTACATCTTCGAC(N)	29, 30
I-179R(a)	GGTGTTGTCGTTGTTCCAGTA GAGGATGTC(N)	
DEV15L	CCGATCATCATGAAGTTYC	
DEV15R	CCAGGTTGGCGCCGATGCC	
Haphapcr1	GTTTTAATCGATTACGCNYTNGTNA AYMGNCCNTAYATG	36
Haphapcr2	CGGGACTATRAADATCCAYTTYT CCATNCKTAGAAAGTT	
P1	ATNGA(CT)TGGGGNTA(CT)CCN	37
P2	(AG)AA(AGT)ATCCA(CT)TT(CT)TCCAT	
B1F	AACTCCTGGGCTTGAAGACA	35
B1R	TCGCAATATGATCACGGCTA	
B2R	ACGGTCCACCAACGTTACAT	

of about 1.3-kb and 1.5-kb DNA fragments containing the 3′ end of *phaC1* and the 5′ end of *phaC2*, respectively (Figure 3). Using the individual PCR fragments as a template in the second-round (semi-nested) PCR with I-179L/I-179R primers would then lead to the specific amplification of an about 0.54-kb segment of the *phaC1* and *phaC2* genes, respectively (Figure 3). Application of this semi-nested PCR to several *Pseudomonas* bacteria led to the refinement of the *phaC*-based primers (I-179La and I-179Ra) to include a single degenerate base at the 3′ end. These two protocols for the rapid and specific identification of class II *pha* genes have provided a simple, convenient, and reliable means to screen bacteria for the presence of these genes. Zhang et al.[31] reported PCR cloning of class II *pha* genes on the basis of the nondegenerate primers derived from the consensus sequences of ORF1, *phaZ* and *phaD* genes of *Pseudomonas pha* loci. Although only *phaC1* (but not *phaC2*) of *Pseudomonas pseudoalcaligenes* HBQ06 and *phaC2* (but not *phaC1*) of *Pseudomonas nitroreducens* 0802 was initially cloned with these primers, subsequent refinement incorporating two-step and touch-down PCR allows the cloning of both the class II *phaC* genes from *Burkholderia caryophylli*.[32]

A broad-specificity PCR procedure for the detection of both the class I and the class II *pha* synthase genes was developed by Sheu et al.[33] The degenerate semi-nested primer pairs (i.e., phaCF1/phaCR4 for the 1st round and phaCF2/phaCR4 for the semi-nested round of PCR; Table 1) were designed on the basis of the highly homologous stretches of the class I and II *pha* genes of 13 Gram-negative bacteria. A sequence alignment study by Rehm[34] on the amino acid sequences of the gene products of 59 *pha* genes of various classes had identified six regions that contain highly conserved amino acid residues. The in-frame translated sequences of the primers phaCF1 (i.e., INKYYILDL in the *W. eutropha* sequence) and phaCF2 (i.e., VFLVSWRNP in *W. eutropha*) are in fact found in the first highly conserved region defined by Rehm, and that associated with primer phaCR4 (i.e., WNYVVDNYL in *W. eutropha*) is in the fourth region (see Figure 2 of ref 34). Sheu et al.'s detection method requires the addition of betaine and dimethylsulfoxide in the PCR reaction mixtures to ensure proper gene amplification. When tested on 19 known PHA-producing bacteria, the first-round PCR failed to detect the *pha* gene in four bacteria, but the semi-nested reaction accurately detected *pha* in all samples. Interestingly, one of these 19 bacterial test samples was *Bacillus megaterium* known to possess class IV *phaC*. Because the nucleic acid sequence of class IV *phaC* is not homologous to those of class I and II genes, it was unfortunate that the about 400-bp amplicon obtained from *B. megaterium* was not sequenced to confirm its classification and for further alignment study. Sheu et al.[33] also applied the method to screen environmental isolates and identified 38 *pha*-positive clones belonging to both Gram-positive and Gram-negative groups. Applying a Nile blue A staining technique to visualize PHA granules showed that 33 of these *pha*-positive isolates stained positively. No information, however, was given regarding the Gram classification of the Nile blue A negative clones, the isolation and composition of PHA from the 33 putative producers of PHA, or the nucleic acid sequences of the PCR amplicons of the 38 *pha*-positive isolates.

PCR detection of the *pha* genes in *Bacillus*, thus, presumably the class IV synthases, was reported by Shamala et al.[35] using nondegenerate primer pairs (B1F/B1R and B1F/B2R; Table 1) designed solely on the basis of the class IV *phaC* gene of *B. megaterium* by employing a primer-design program. Because of the simplistic approach by which these primers were designed, however, a global detection of class IV *pha* genes should not be expected. In fact, these investigators used a number of *Bacillus* species (i.e., *B. megaterium*, *B. amylovorans*, *B. laterosporus*, *B. circulans*, *B. subtilis*, *B. cereus*, and *B. brevis*) as standard samples to verify their method, but they only reported a definitive positive PCR amplification of *phaC* with the *B. megaterium* sample. Strangely, while the majority of their standard species of *Bacillus* (e.g., *B. circulans* and *B. brevis*) did not produce the expected amplicons (as assayed by PCR) or PHA (as determined by solvent extraction and FT-IR analysis), their environmental isolates belonging to these species were shown to react positively to the PCR assay and produce PHA (see Table 1 of ref 35). We had performed a sequence alignment analysis of known *Bacillus phaC* sequences [i.e., those of *B. megaterium* (GenBank gi:13812266), *B. thuringiensis* (gi:49328240), three strains of *B. anthracis* (gi:30255149, 50082967, 49176966), two strains of *B. cereus* (gi:29894935, 42736135), and *Bacillus* sp. INT005 (gi:27348111)] and found that the sequence of the *B. megaterium phaC* has the least sequence matches to the other seven query sequences (data not shown). It was, thus, unclear how Shamala et al.'s nondegenerate primers were able to detect *pha* genes in their environmental isolates classified as *B. sphaericus*, *B. circulans*, and *B. brevis* and why the same primers did not unequivocally yield PCR products in the standard species of these organisms they used. It should be

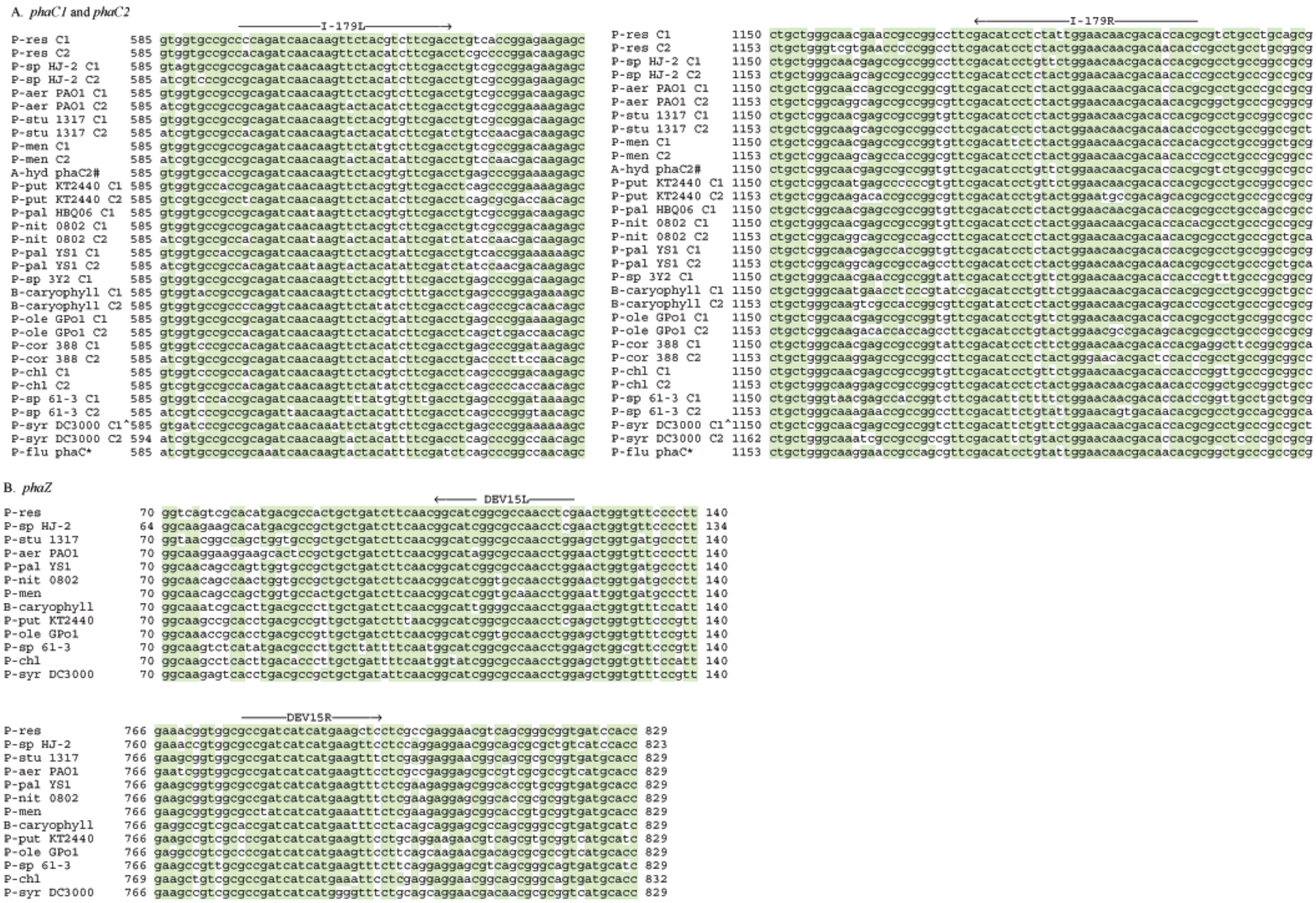

Figure 2. Nucleic acid sequence alignment of class II *phaC1* and *phaC2* (panel A) and *phaZ* (panel B) genes, showing the highly conserved regions containing the primer-binding sites. The Clone Manager Suite program (version 7, Sci Ed Software, Durham, NC) was used to perform the Multi-Way alignment. (#) Confusing annotations in the GenBank entry, where the title indicates *phaC1* study but the sequence was attributed to *phaC2*; (∧) the sequence contains a genuine point mutation (not sequencing error) that causes premature translation termination; (*) not specified as *phaC1* or *phaC2.* Abbreviations: P-pal HBQ06, *P. pseudoalcaligenes* HBQ06 (gi:13346167); A-hyd, *Aeromonas hydrophila* (gi: 55140654); P-syr DC3000, *Pseudomonas syringae* pv. tomato str. DC3000 (gi:28855325); B-caryophyll, *B. caryophylli* (gi:15028441); P-aer PAO1, *P. aeruginosa* PAO1 (gi:9951346); P-pal YS1, *P. pseudoalcaligenes* YS1 (gi:15421134); P-chl, *Pseudomonas chlororaphis* (formerly *Pseudomonas aureofaciens*) (gi:17402509); P-men, *P. mendocina* (gi:20086522); P-nit 0802, *P. nitroreducens* 0802 (gi:19589601); P-ole GPo1, *P. oleovorans* Po1 (gi:151441); P-put KT2440, *P. putida* KT2440 (gi:26557037); P-res, *P. resinovorans* (gi:10835918); P-sp 61−3, *Pseudomonas* sp. 61−3 (gi:4062966); P-sp HJ-2, *Pseudomonas* sp. HJ-2 (gi:34452170); P-stu 1317, *Pseudomonas stutzeri* 1317 (gi:30721689); P-sp 3Y2, *Pseudomonas* sp. 3Y2 (gi:54610879); P-cor, *P. corrugata* 388 (gi:51234088 and 51234090); P-flu, *Pseudomonas fluorescens* (gi:37925924); and C1 and C2, *phaC1* and *phaC2*, respectively.

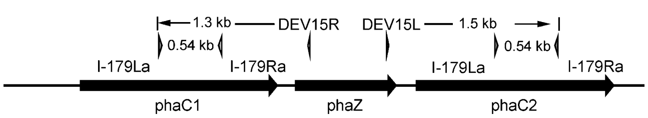

Figure 3. Semi-nested PCR of class II *phaC1* and *phaC2* genes. Using the *P. resinovorans pha* locus as an example, the first-round PCR using the I-179L(a)/DEV15R primer pair yielded a 1.3-kb product, which on the second-round (semi-nested) PCR with I-179L(a)/I-179R-(a) primer pair then resulted in a specific 0.54-kb *phaC1* fragment. Likewise, first-round PCR using DEV15L/I-179R(a) primers produced a 1.5-kb fragment, which on the second-round PCR with I-179L(a)/I-179R(a) primer pair yielded the specific 0.54-kb *phaC2* fragment.

noted that in the sequence alignment analysis just mentioned, the *phaC* genes of *B. thuringiensis* and the three strains of *B. anthracis* are identical (100% identity) and exhibit 95−97% sequence match to those of *B. cereus* (both strains) and *Bacillus* sp. INT005. Perhaps the class IV *phaC* sequences can be divided into two subgroups on the basis of the extent of nucleic acid sequence matches: the *B. megaterium* subgroup (including *B. sphaericua*, *B. circulans*, and *B. brevis*) and the *B. cereus* subgroup (including *B. anthracis*, *B. thuringiensis*, and the INT005 isolate). In this respect, it would be of value to have the sequences of the *phaC* genes (or at least of the PCR fragments amplified by Shamala et al.) of the other members of the proposed *B. megaterium* subgroup.

In their study of the class III *pha* genetics of cyanobacteria and sulfate-reducing bacteria (SRB), Hai et al.[36,37] developed PCR protocols to screen for the related *phaC* genes. The degenerate primers, Haphapcr1 and Haphapcr2 (Table 1), used in the study of cyanobacteria were based on the highly conserved sequences of the class III *phaC* genes of, among others, *Allochromatium vinosum*, *Thiocystis violacea*, and *Synechocystis* sp. PCC 6803. More specifically, Haphapcr1 and Haphapcr2 correspond to amino acid residues 85-97 and 263-275, respectively, of the *phaC* gene product of *Synechocystis* sp. PCC 6803.[36] The Haphapcr1/2-based PCR protocol, however, failed to correctly identify three strains (*Cyanothece* sp. PCC7424 and PCC7428 and *Stanieria* sp.

PCC7437) as PHB-producing strains out of the nine screened. The PCR primers (P1 and P2, Table 1) used in the SRB study were based on the highly conserved amino acid sequences (VNRPYM and MEKWIF) contained inside the same amino acid residues of *Synechocystis* sp. PCC 6803 mentioned earlier. Although the report indicated successful PCR amplification of *phaC* gene fragments from certain SRB, details of this result were not presented or discussed.

Applications

PCR methods for *phaC* detection are powerful first-line screening means to identify potential PHA-producing organisms. This is especially the case when PHA synthesis is under any unknown regulation and, thus, direct PHA assays (such as lipophilic-dye staining or FT-IR methods) are not applicable when cells are not producing or accumulating the polymers. There could also be instances where a gene may be present but the product is clearly not produced, that is, a silent, pseudo- or iso-gene. Such cases would then provide excellent systems for the study of the regulation (whether at the genetic or the metabolic level) of PHA synthesis. Although the availability of increasing numbers of genomic sequences provides an important avenue for the study of *pha* genetics, it remains a fact that the genomes of still many other bacteria with interesting metabolic backgrounds have not been sequenced. PCR detection methods will, thus, continue to play an important role in the identification and subsequent characterization of PHA-producing bacteria.

The *phaC* gene fragment resulting from the PCR assays provides a valuable entry point toward the complete cloning of the entire *phaC* gene and subsequently the associated gene assembly. One usage of this gene fragment in the cloning of *phaC* and its associated genes is to serve as a probe in the screening of the genomic library of the organism. Because the fragment is a bona fide part of the *phaC* gene, a strong and specific hybridization can be expected and, thus, greatly facilitates the process of a correct identification of the positive clones. The other application of the fragment is to provide sequence information for the cloning of the *pha* genes. The sequence of the PCR product can be readily determined either directly using the amplicon as a template or through its subcloning into a cloning vector. In the first instance, the primers used in the PCR can often serve as the sequencing primers as well. In the latter case, various universal sequencing primers appropriate for the particular cloning vector may be used. Once the sequence is determined, one of the many sequence extension approaches could be used to obtain the sequences of the *phaC* and other genes in its assembly. An inverse PCR approach,[38] for example, has been used to complete the cloning of class III *phaC* genes of cyanobacteria[36] and SRB[37] and of the class I *phaC* genes of *P. oleovorans* NRRL B-778 (GenBank gi:33465809; Solaiman, unpublished data) and NRRL B-14682 (GenBank gi:39607630; Solaiman, unpublished data). The PCR-based genomic DNA walking approach is another powerful means to clone the *pha* operon. In this approach, conventional PCR and, if necessary, the subsequent semi- or fully nested PCR are performed using primer pairs consisting of a target-specific and a random primer. Numerous strategies and refinements of this approach have been developed over the years.[39] The sequence information of the *phaC* gene fragment obtained from the PCR detection plays a crucial role in the designing of the target-specific primers in these strategies. The cloning of the class II *pha* locus of *Pseudomonas resinovorans* NRRL B-2649 serves as an example of the application of this approach in which vectorette PCR[40] and uneven PCR[41] techniques were used.[42] The application of another variation of this approach, that is, the annealing-control-primer DNA walking,[43] led to the sequence characterization of the entire *phaC1* and *phaC2* genes of *Pseudomonas corrugata* (GenBank gi:51234090 and 51234088; Solaiman, unpublished data). The third approach to obtain the entire *phaC* gene and its associated gene assemblage can be adopted on the basis of Garcia et al.'s strategy.[44] These authors reported a method to clone large gene assemblages associated with known sequences. The strategy is based on single crossover homologous recombination event occurring in the chromosome at the location of the genes of known sequence. Subsequent selective restriction digestion allows the excision of the gene assemblage associated with the known sequences. Garcia et al.[44] proceeded to demonstrate the applicability of their strategy by cloning the gene assemblages of the phenylacetic acid catabolic pathway and of the PHA-synthesis pathway of *P. putida* U. Again, using the sequence information of the *phaC* fragment obtained from the PCR detection protocols, it is conceivable to apply Garcia et al.'s strategy to clone the entire gene assemblage of the PHA biosynthesis.

Another underexplored application using the sequence information of the PCR-amplified *phaC* fragment is the characterization of bacterial isolates. Solaiman[30] applied this method to clearly differentiate the three isolates of *P. oleovorans* (i.e., NRRL B-778, B-14682, and B-14683) as distinct strains, leading to subsequent physiological characterization of each strain as a scl-, mcl-, or scl-and-mcl-PHA-producing organism.[45,46] Furthermore, initial comparison of five strains of *P. corrugata* (i.e., ATCC 29736, 313, 388, 412, and 717) isolated from different locations and plants showed that their respective PCR-amplified *phaC1* and *phaC2* PCR fragments have identical nucleic acid sequences, suggesting that the acquisition of the *pha* locus in *P. corrugata* is a recent event in its evolutionary history.[30] A subsequent study of 56 strains of *P. corrugata* and 21 strains of a closely related species, *Pseudomonas mediterranea*,[47] with respect to the sequences of the PCR fragments of their *phaC1*/*phaC2* genes support the establishment of *P. mediterranea* as a distinct taxonomic group (Solaiman and Catara, unpublished data).

Conslusion

Rapid PCR methods have been developed to detect the four classes of *phaC* genes. These methods provide powerful screening tools for the identification of potential PHA-producing organisms. The PCR fragment also serves as an entry point for the complete cloning and characterization of the *pha* gene locus. Microbial characterization and classification can also benefit from the sequence analysis of the PCR-amplified *phaC* fragment. Future development of a universal PCR protocol for the detection of all classes of *phaC*'*s* could lead to the identification of unknown PHA synthases from environmental samples containing unculturable organisms.

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